Breeding Science 63: 400–406 (2013)
doi:10.1270/jsbbs.63.400

Efficient haploid and doubled haploid production from unfertilized ovule culture of gentians (*Gentiana* spp.)

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Factors affecting reliable plant regeneration from unfertilized ovule culture of gentians (*Gentiana* spp.) were examined. Cold pretreatment (4°C) of flower buds enhanced or maintained production of embryo-like structure (ELS). When 43 genotypes were surveyed in two different labs, 40 of them produced ELSs ranging from 0.01 to 26.5 ELSs per flower bud. No ELSs could be obtained in three genotypes. A significant correlation ($r = 0.64$) was observed between the number of ELS per flower and the frequency of responding flower buds. Eight genotypes of *G. triflora*, which were used as common materials in two different labs, produced ELSs in both labs. The ploidy levels of a total of 1,515 regenerated plantlets were determined, revealing that the majority of these plants consisted of haploids (57.9%) and diploids (34.3%). However, the frequency of haploids and diploids was different between *G. triflora* and *G. scabra*, and *G. triflora* showed higher frequencies of haploids than *G. scabra*. When haploids were treated with oryzalin for chromosome doubling, diploids and tetraploids were obtained. These results demonstrate that the unfertilized ovule culture technique of gentians is a powerful tool for obtaining haploids and DHs because of its reproducible and reliable nature and application to a wide range of genotypes.

Key Words: *Gentiana triflora*, *G. scabra*, unfertilized ovule culture, genotype, haploid, doubled haploid.

Introduction

Some species of *Gentiana* have been used as ornamental plants in Europe and Asia. Although gentians are commonly used in rock gardens and garden borders in Europe, they are one of the most important plants for cut-flower and pot-plant use in Japan. Both *G. triflora* and *G. scabra*, which grow wild in Japan, have been cultivated commercially as ornamental plants and many F_1 and clonal cultivars have been bred (Takahata *et al.* 1995, Yoshiike 1992). Although homozygous parental lines are indispensable for F_1 hybrid breeding, it is difficult to obtain homozygous lines owing to their intense inbreeding depression.

Recently, successful production of haploids and doubled haploids (DHs) has been reported in male and female gametophytic cells by *in vitro* culture of anthers (androgenesis) in *G. triflora* and unfertilized ovules (gynogenesis) in some species of *Gentiana* (*G. triflora*, *G. scabra* and their hybrids), respectively (Doi *et al.* 2010, 2011, Pathirana *et al.* 2011). Doi *et al.* (2011) indicated that unfertilized ovule culture has more advantages than anther culture from the point of view of embryogenesis efficiency, limited influence of donor plant genotype and a high frequency of haploids and DHs production. However, this conclusion was inferred from results derived from limited plant materials (four genotypes). For practical use of unfertilized ovule culture for breeding, it is important to determine whether this technique can be applied to a number of genotypes and used with high reproducibility in other labs.

Cold pretreatment of flower buds or inflorescences before culture has been reported to enhance the frequency of embryogenesis not only through androgenesis but also through gynogenesis in several species (Chen *et al.* 2011, Sopory and Munshi 1996). In addition, the storage of buds in a low temperature for certain periods can avoid the concentration of culture work over a limited time (Sato *et al.* 2002, Takahashi *et al.* 2012).

In this study, to clarify the genotypic variation in embryo-like structure (ELS) production from unfertilized ovule culture of gentians and the reproducibility of the results obtained by using this technique, we investigated the ability of ELS production and plant regeneration on 43 genotypes at two different labs. In addition, the effect of cold pretreatment on unfertilized ovule culture and the optimum

Communicated by R. Ohsawa
Received July 23, 2013. Accepted September 15, 2013.
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condition of chromosome doubling of haploid plants were examined.

Materials and Methods

Plant materials

Forty-three genotypes of gentians, which are listed in Table 1, were used in this study. They consisted of 12 cultivars and 31 lines of *G. triflora* ($2n = 26$), *G. scabra* ($2n = 26$), *G. triflora* var. *japonica* f. *montana* ($2n = 26$) and their hybrids (*G. triflora* × *G. scabra*, *G. scabra* × *G. triflora*, *G. triflora* var. *japonica* f. *montana* × *G. triflora*). These materials were grown in experimental fields and in a greenhouse at Hachimantai City Floricultural Research and Development Center, Hachimantai, Iwate, Japan and at Iwate Agricultural Research Center (IARC), Kitakami, Iwate, Japan.

Unfertilized ovule culture and plantlet regeneration

Unfertilized ovule culture was carried out as described by Doi *et al.* (2011) at two different labs, Iwate University, Morioka and IARC, Kitakami. Flower buds, which were in developmental stage 4 to stage 6 (Doi *et al.* 2011), were taken from the donor plants. After the petals and stamens were removed, the pistils were surface-sterilized in 70% ethanol for 30 s, followed by sodium hypochlorite solution (2% active chlorine) for 15 min and then rinsed three times with sterile distilled water (5 min each time). Ovules excised from a pistil were cultured on a 60-mm plastic Petri dish containing 0.8% agar-solidified 1/2 NLN medium (Takahata and Keller 1991) supplemented with 10% sucrose. The Petri dishes were maintained at 25°C under dark condition. There were at least three plates per experiment and each experiment had at least three independent replicates. Statistical analysis was performed using the computer program JMP 8.0 (SAS Institute Inc.).

The ELSs developed from ovules were transferred to modified agar (1.0%)-solidified MS medium (Murashige and Skoog 1962) with the concentration of major salts reduced by 50% (1/2MS) and supplemented with 3% sucrose and 1.0 mg/l GA_3 and incubated at 20°C with a 16 h/day photoperiod. Regenerated plants were grown in vermiculate and then transferred to soil in a greenhouse.

Cold pretreatment

The effect of cold pretreatment for unfertilized ovule culture was investigated using four genotypes (*G. triflora* cv. Ashiro-no-Aki, *G. scabra* line 17-260, *G. triflora* × *G. scabra* lines 17-386 and 17-488), which already demonstrated the ability of ELS production (Doi *et al.* 2011). After anthers were removed from each flower, inflorescences were stored at low temperature (4°C) for 3, 7 and 14 days in the dark. After this treatment, unfertilized ovules were cultured as described above.

Ploidy level determination by flow cytometry

The ploidy level of regenerated plantlets was analyzed using Partec CyFlow PA (Partec GmbH, Germany) and Cell Lab Quanta SC (Beckman Coulter, USA) at Iwate University and IARC, respectively. Materials for ploidy determination were prepared from young leaf (approx. 25 mm^2) of regenerated plants. Sample preparation and

Table 1. List of cultivars/lines used in this study

Species Cultivar/line	Type	Source
G. triflora		
Ashiro-no-aki	F_1 Hybrid	Hachimantai
Cust	F_1 Hybrid	Kitakami
Giovanni	F_1 Hybrid	Kitakami
Goku-wase2	F_1 Hybrid	Kitakami
Homoi	F_1 Hybrid	Kitakami
Ihatovo	F_1 Hybrid	Kitakami
Iwate	F_1 Hybrid	Kitakami
Marjel	F_1 Hybrid	Kitakami
Maciry	F_1 Hybrid	Kitakami
06-6	Sib-cross line	Kitakami
06-8	Sib-cross line	Kitakami
05-01B	Sib-cross line	Kitakami
05-02B	Sib-cross line	Kitakami
05-03B	Sib-cross line	Kitakami
05-04B	Sib-cross line	Kitakami
05-05B	Sib-cross line	Kitakami
05-07B	Sib-cross line	Kitakami
05-12B	Sib-cross line	Kitakami
05-17B	Sib-cross line	Kitakami
05-571	Sib-cross line	Kitakami
06-512YRy	Sib-cross line	Kitakami
06-65SPB	Sib-cross line	Kitakami
07-520	Sib-cross line	Kitakami
07-533	Sib-cross line	Kitakami
G. scabra		
17-260	F_1 Hybrid	Hachimantai
Alta	F_1 Hybrid	Kitakami
G. triflora var. *japonica* f. *montana*		
AZ1 early1	Clonal line	Hachimantai
G. triflora × *G. scabra*		
14-218-13	Clonal line	Hachimantai
14-218-14	Clonal line	Hachimantai
14-218-20	Clonal line	Hachimantai
14-218-21	Clonal line	Hachimantai
14-218-3	Clonal line	Hachimantai
14-218-30	Clonal line	Hachimantai
17-386	F_1 Hybrid	Hachimantai
17-488	F_1 Hybrid	Hachimantai
G. scabra × *G. triflora*		
LBbc	F_1 Hybrid	Kitakami
Polarno blue	F_1 Hybrid	Kitakami
07-119B	Sib-cross line	Kitakami
07-119P	Sib-cross line	Kitakami
07-119W	Sib-cross line	Kitakami
07-123B	Sib-cross line	Kitakami
07-123P	Sib-cross line	Kitakami
G. triflora var. *japonica* f. *montana* × *G. triflora*		
17-771	F_1 Hybrid	Hachimantai

Table 2. Effect of cold pretreatment (4°C) on ELS production in unfertilized ovule culture of gentians

Species Cultivar/line	Cold pretreatment (day)	No. of cultured flower buds	Frequency of responding flower buds (%)	ELS induction	
				No. of ELS	ELS per flower bud*
G. triflora	0	64	62.6	106	1.66 b
cv. Ashiro-no-Aki	3	88	66.2	162	1.84 b
	7	83	73.5	570	6.87 a
	14	102	53.7	210	2.06 b
G. scabra	0	56	41.7	53	0.95 b
17-260	3	57	60.5	107	1.88 b
	7	66	65.6	107	1.62 b
	14	58	56.0	111	1.91 b
G. triflora × *G. scabra*	0	76	7.2	8	0.11 b
17-386	3	66	5.5	6	0.09 b
	7	66	0.0	0	0.00 b
	14	85	5.2	4	0.05 b
17-488	0	83	67.2	111	1.34 b
	3	83	57.3	95	1.14 b
	7	89	68.2	177	1.99 b
	14	98	78.9	195	1.99 b

*Means within columns followed by different letters are different at the 0.05 level by the Tukey-Kramer's test.

Table 3. Effect of genotypes on ELS production in unfertilized ovule culture of gentians at Iwate University

Species	Cultivar / line	No. of cultured flower buds	Frequency of responding flower buds (%)	ELSs production	
				No. of ELS	ELS per flower bud*
G. triflora	Ashiro-no-Aki	185	63.6	780	4.22 cd
	Cust	112	34.6	331	2.96 cd
	Ihatovo	58	85.6	1539	26.53 a
	Marjel	121	56.5	438	3.62 cd
	Maciry	112	59.1	643	5.74 bcd
	06-6	160	68.6	1007	6.29 bcd
	05-01B	44	20.6	34	0.77 cd
	05-02B	62	57.6	90	1.45 cd
	05-03B	93	52.0	196	2.11 cd
	05-04B	52	44.1	80	1.54 cd
	05-05B	66	94.1	697	10.56 bc
	05-07B	48	50.0	73	1.52 cd
G. scabra	17-260	124	60.8	218	1.76 d
G. triflora var. *japonica* f. *montana*	AZ1 early1	110	6.2	14	0.13 cd
G. triflora × *G. scabra*	17-386	151	2.6	4	0.03 d
	17-488	187	73.5	372	1.99 cd
	14-218-3	99	23.1	24	0.24 d
	14-218-13	79	19.9	17	0.22 d
	14-218-14	78	46.7	72	0.92 d
	14-218-20	70	66.3	138	1.97 cd
	14-218-21	101	61.9	139	1.38 d
	14-218-30	81	70.0	218	2.69 cd
G. triflora var. *japonica* f. *montana* × *G. triflora*	17-771	122	87.1	1485	12.17 b

*Means within columns followed by the different letters are significantly different at the 0.05 level by Tukey-Kramer's test.
The statistical analysis was carried out on the whole data set.

measurement of the ploidy level were performed according to Doi *et al.* (2010) in the former analysis and Mishiba *et al.* (2009) in the latter.

Chromosome doubling treatment

Chromosome doubling of haploids was performed as described by Morgan *et al.* (2003) with minor modification. Haploid plants were maintained in 1/2 MS medium, and

Table 4. Effect of genotypes on ELS production in unfertilized ovule culture of gentians at Iwate Agricultural Research Center (IARC)

Species	Cultivar/line	No. of cultured flower buds	Frequency of responding flower buds (%)	ELS production	
				No. of ELSs	ELSs per flower bud*
G. triflora	Cust	262	36.3	293	1.12 f
	Giovanni	42	52.4	73	1.74 def
	Goku-wase2	233	73.8	1015	4.36 b
	Homoi	11	63.6	19	1.73 bcdef
	Ihatovo	97	41.2	161	1.66 ef
	Iwate	33	27.3	11	0.33 ef
	Marjel	136	6.6	17	0.13 f
	Maciry	130	38.5	139	1.07 ef
	06-6	22	68.2	112	5.09 bcd
	06-8	7	71.4	144	20.57 a
	05-04B	25	16.0	4	0.16 ef
	05-05B	57	26.3	23	0.40 f
	05-07B	63	9.5	6	0.10 f
	05-12B	14	0.0	0	0.00 ef
	05-17B	107	22.4	26	0.24 f
	05-571	80	7.5	6	0.08 f
	06-512YRy	106	70.8	286	2.70 cde
	06-65SPB	94	18.1	27	0.29 f
	07-520	112	25.9	53	0.47 f
	07-533	114	66.7	508	4.46 bc
G. scabra	Alta	86	16.3	75	0.87 ef
G. triflora × *G. scabra*	LBbc	75	0.0	0	0.00 f
	Polarno blue	134	0.0	0	0.00 f
G. scabra × *G. triflora*	07-119B	67	40.3	57	0.85 ef
	07-119P	48	52.1	54	1.13 ef
	07-119W	56	17.9	13	0.23 f
	07-123B	97	1.0	1	0.01 f
	07-123P	99	16.2	53	0.54 f

*Means within columns followed by different letters are significantly different at the 0.05 level by the Tukey-Kramer's HSD test.
The statistical analysis was carried out on the whole data set.

subcultured to propagation (Pr) medium (1/2MS medium supplemented with 1.0 mg/l BA and 1.0 mg/l GA_3) to increase shoot numbers for chromosome doubling treatment. After 4 weeks of culture, elongated shoots containing axillary buds were cut and subcultured to Pr medium containing 50 μM oryzalin for 1, 2, 3 and 4 weeks. Their explants were then cultured in Pr medium for 6 weeks and elongated axillary shoots were transferred to Pr medium without BA. After 4 weeks of culture, normal plantlets were subcultured to 1/2MS medium and their ploidy level was determined by flow cytometry.

Results

Effect of cold pretreatment

The effect of cold pretreatment of plant materials on ELS production was examined using four genotypes. Cold pretreatment produced more ELSs than non-treatment in three genotypes except for *G. triflora* × *G. scabra* line 17-386 (Table 2). In particular, cold pretreatment for 7 and 14 days tended to exhibit a higher response, and a significant increase was shown in *G. triflora* cv. Ashiro-no-Aki treated at low temperature for 7 days.

Effects of genotypes and labs

The effect of genotypes on production of ELSs from unfertilized ovule culture was examined independently at Iwate Univ. and IARC and the results are shown in Tables 3, 4, respectively. Although genotypic variations on ELS production are shown, almost all genotypes produced ELSs in both labs except for 3 genotypes. The plant materials used at Iwate Univ. were treated at low temperature for 7–14 days and those used at IARC were not treated at low temperature. All 23 genotypes used at Iwate Univ. produced ELSs ranging from 26.5 ELSs per flower bud of *G. triflora* cv. Ihatovo to 0.03 of *G. triflora* × *G. scabra* line 17-386 (Table 3). In IARC, of 28 genotypes used, 25 produced ELSs ranging from 20.6 ELSs per flower bud of *G. triflora* line 06-6 to 0.01 of *G. scabra* × *G. triflora* line 07-123B (Table 4). Three genotypes, *G. triflora* 05-12B, *G. tiflora* × *G. scabra* LBbc and Polarno blue, produced no ELSs. The frequency of responding flower buds was significantly related to the number of ELS per flower bud ($r = 0.64$ based on merged data of Tables 3, 4).

Eight genotypes of *G. triflora* cvs./lines, i.e., Cust, Ihatovo, Marjel, Maciry, 06-6, 05-04B, 05-05B and 05-07B, which were used as common materials in two different labs,

Table 5. Determination of ploidy level of plantlets derived from unfertilized ovule culture of gentian

Cultivar/line	Number of analyzed plants	No. of plants (%)						
		x	*2x*	*3x*	*4x*	*5x*	*6x*	Chimeric plant
G. triflora								
Ashirono-aki	70	36 (51.4)	27 (38.6)	3 (4.3)	0	0	2 (2.9)	2 (2.9)
Cust	53	36 (67.9)	15 (28.3)	0	1 (1.9)	0	0	1 (1.9)
Giovanni	19	14 (73.7)	5 (26.3)	0	0	0	0	0
Goku-wase2	289	170 (58.8)	112 (38.8)	3 (1.0)	4 (1.4)	0	0	0
Homoi	5	5 (100)	0	0	0	0	0	0
Ihatovo	87	53 (60.9)	21 (24.1)	4 (4.6)	4 (4.6)	0	0	5 (5.7)
Iwate	1	1 (100)	0	0	0	0	0	0
Marjel	35	17 (48.6)	14 (40)	1 (2.9)	2 (5.7)	0	0	1 (2.9)
Maciry	107	68 (63.7)	31 (29.0)	0	3 (2.8)	0	0	5 (4.7)
06-6	99	57 (57.6)	33 (33.3)	5 (5.1)	3 (3.0)	0	0	1 (1.0)
06-8	25	17 (68.0)	7 (28.0)	1 (4.0)	0	0	0	0
05-01B	7	4 (57.1)	1 (14.3)	0	0	0	0	2 (28.6)
05-02B	9	7 (77.8)	2 (22.2)	0	0	0	0	0
05-03B	55	36 (65.5)	17 (30.9)	1 (1.8)	1 (1.8)	0	0	0
05-04B	19	10 (52.6)	8 (42.1)	0	0	0	0	1 (5.3)
05-05B	16	8 (50.0)	7 (43.8)	1 (6.2)	0	0	0	0
05-07B	10	3 (30.0)	6 (60.0)	0	0	0	0	1 (10.0)
05-17B	2	2 (100)	0	0	0	0	0	0
06-512Yry	96	91 (94.8)	4 (4.2)	1 (1.0)	0	0	0	0
06-65SPB	5	5 (100)	0	0	0	0	0	0
07-520	7	6 (85.7)	1 (14.3)	0	0	0	0	0
07-533	128	114 (89.1)	11 (8.6)	2 (1.6)	1 (0.8)	0	0	0
Total	1144	760 (66.4)	322 (28.1)	22 (1.9)	19 (1.7)	0	2 (0.2)	19 (1.7)
G. scabra								
17-260	176	42 (23.9)	102 (58)	11 (6.3)	16 (9.1)	0	0	5 (2.8)
Alta	33	10 (30.3)	23 (69.7)	0	0	0	0	0
Total	209	52 (24.9)	125 (59.8)	11 (5.3)	16 (7.7)	0	0	5 (2.4)
G. triflora var. *japonica* f. *montana*								
AZ1 early1	1	0	1 (100)	0	0	0	0	0
G. triflora × *G. scabra*								
14-218-20	4	2 (50.0)	2 (50.0)	0	0	0	0	0
14-218-21	9	1 (11.1)	7 (77.8)	0	0	0	0	1 (11.1)
14-218-30	2	0	2 (100)	0	0	0	0	0
17-386	3	1 (33.3)	2 (66.7)	0	0	0	0	0
17-488	37	7 (18.9)	28 (75.7)	1 (2.7)	1 (2.7)	0	0	0
Total	55	11 (20)	41 (74.5)	1 (1.8)	1 (1.8)	0	0	1 (1.8)
G. scabra × *G. triflora*								
07-119B	19	10 (52.6)	6 (31.6)	1 (5.3)	1 (5.3)	1 (5.3)	0	0
07-119P	8	5 (62.5)	3 (37.5)	0	0	0	0	0
07-119W	1	0	1 (100)	0	0	0	0	0
Total	28	15 (53.6)	10 (35.7)	1 (3.6)	1 (3.6)	1 (3.6)	0	0
G. triflora var. *japonica* f. *Montana* × *G. triflora*								
17-771	78	39 (50.0)	25 (32.1)	9 (11.5)	2 (2.6)	0	0	3 (3.8)
Total	1515	877 (57.9)	524 (34.6)	44 (2.9)	39 (2.6)	1 (0.06)	2 (13.2)	28 (1.85)

produced ELSs in both labs. However, the frequency of ELS production in Iwate Univ. (a mean value of 7.35 ELSs per flower) was higher than those in IARC (1.22 ELSs per flower). Such a trend was also found in results obtained from data of all genotypes (a mean value of 3.95 ELSs per flower in the former vs. 1.80 ELSs per flower in the latter).

Ploidy level of regenerated plants

The ploidy levels of a total of 1,515 regenerated plantlets, which were randomly chosen from regenerated plantlets, were determined by flow cytometry (Table 5). The majority of these plants consisted of haploids (57.9%) and diploids (34.3%). A higher frequency of haploids was obtained in *G. triflora* (66.4% of haploids and 28.1% of diploids), while a higher frequency of diploids was obtained in *G. scabra*

Table 6. Effect of oryzalin treatment periods on chromosome doubling in gentians

Species (No. of genotype used)	Treatment period (w)	No. of plants examined	No. of plants (%)					
			x	2*x*	4*x*	*x* + 2*x*	*x* + 4*x*	2*x* + 4*x*
G. triflora	0	24	24 (100)	0	0	0	0	0
(5 genotypes[a])	1	46	13 (28.3)	11 (23.9)	20 (43.5)	0	0	2 (4.3)
	2	24	3 (12.5)	9 (37.5)	9 (37.5)	2 (8.3)	0	1 (4.2)
	4	28	4 (14.3)	9 (32.1)	14 (50.0)	0	0	1 (3.6)
G. scabra	0	25	25 (100)	0	0	0	0	0
(17-260 line)	1	42	26 (61.9)	8 (19.0)	6 (14.3)	1 (2.4)	0	1 (2.4)
	2	29	20 (69.0)	5 (17.2)	2 (6.9)	2 (6.9)	0	0
	4	29	24 (82.8)	1 (3.4)	2 (6.9)	0	1 (3.4)	1 (3.4)
G. triflora var. *japonica*	0	10	10 (100)	0	0	0	0	0
f. *montana* × *G. triflora*	1	11	6 (54.5)	2 (18.2)	3 (27.3)	0	0	0
(17-771 line)	2	12	10 (83.3)	2 (16.7)	0	0	0	0
	4	2	2 (100)	0	0	0	0	0

[a] Five genotype consist of cv. Ashiro-no-Aki, cv. Maciry, 06-6, 05-03B and 05-05B.

(24.9% of haploids and 59.8% of diploids). Besides diploid and haploid, a higher ploidy from triploid to hexaploid and chimera were found in the several genotypes.

Chromosome doubling treatment

In order to obtain DH plants effectively, haploids of *G. triflora*, *G. scabra* and *G. triflora* var. *japonica* f. *montana* × *G. triflora* were treated by 50 μM oryzalin. This treatment produced diploid plants at various frequencies in all species ranging from 37.5% of *G. triflora* to 16.7% of *G. triflora* var. *japonica* f. *nontana* × *G. scabra* (Table 6). In addition to diploids, tetraploids and mixoploids were obtained in all species used. Optimum periods of treatment depended on species. In *G. triflora*, treatment for 2–4 weeks gave the highest production of diploid and tetraploid (82%), in contrast, in two other species, treatment for 1 week showed the highest frequency of diploid and tetraploid production.

Discussion

Cold pretreatment was found to be beneficial for production of ELSs from unfertilized ovule culture of gentians in the present study. These results are consistent with those of unfertilized ovule/ovary culture in *Beta vulgaris* (Gurel *et al.* 2000, Lux *et al.* 1990) and *Triticum durum* (Sibi *et al.* 2001). On the other hand, no positive influences of cold pretreatment on gynogenic response were reported in *Cucrubita pepo* (Metwally *et al.* 1998) and *Guizotia abyssinica* (Bhat and Murthy 2007). In *G. triflora*, Pathirana *et al.* (2011) also recommended cold pretreatment (4°C for 48 h) on anther and ovary culture, though they did not show detailed data. Our results indicate that cold pretreatment for 1–2 weeks is beneficial for effective induction of ELSs in gentians, and as mentioned in Introduction, such pretreatment could be useful for avoidance of the concentrated ovule culture work within a limited time.

The donor genotype is known to be one of the most important factors in various tissue culture systems. In unfertilized ovule culture, the genotypic effect has also been reported in several species (reviewed by Bohance 2009, Chen *et al.* 2011). The present study using 43 genotypes including *G. triflora*, *G. scabra*, *G. triflora* var. *japonica* f. *montana* and their interspecific hybrids showed that 40 genotypes produced ELSs and regenerated plantlets in spite of genotypic variations in their frequency. Our previous report which used four genotypes indicated that although genotypic variations are present, unfertilized ovule culture was affected less by genotypes compared with that of anther culture (Doi *et al*. 2011). The present study reinforced the effectiveness of the unfertilized ovule culture for production of haploids and DHs, and revealed that although there is the effect of genotype in the frequency of ELS production, unfertilized ovule culture could be utilized for production of haploids and DHs among a wide range of gentian genotypes. Such genotypic variations and applicability of a wide range of genotypes are reported in gynogenesis of several plants such as onion, sugar beet and carrot (Bohanec and Jakse 1999, Geoffriau *et al.* 1997, Gurel *et al.* 2000, Kiełkowska and Adamus 2010).

Reproducibility of the results obtained by tissue culture techniques is a fundamental requirement in plant breeding as well as basic studies. To test such reproducibility and reliability of our culture system, we carried out unfertilized ovule culture in two different labs, at Iwate Univ. and at IARC. Our results revealed that all eight genotypes used as common materials in these two labs demonstrated the production of ELSs in both labs as well. In addition, other genotypes, which were used in each lab, also produced ELSs. These results indicated that unfertilized ovule culture system is reproducible and stable for production of haploids and DHs in gentians. On the other hand, the frequency of ELS production at Iwate Univ. was higher than that at IARC. Such a higher frequency of production at Iwate Univ. might have been due to the application of cold pretreatment of materials and/or the involvement of more experienced

researchers on unfertilized ovule culture.

A large number of regenerated plantlets, which were obtained in this study, consisted of haploids, diploids, polyploids and chimeric ones and the majority of these plants were haploids (57.8%) and diploids (34.6%). However, their frequencies were different between species. More than half of the regenerants (66.4%) were haploids in *G. triflora*, whereas more than half of the regenerants (59.8%) were diploids in *G. scabra*. Such results were consistent with those of previous reports (Doi *et al.* 2011). Although we did not investigate whether diploid plants are DHs or not, we are certain that the majority of diploid plants are DHs, because we have already reported that 96% of diploids plants obtained via gynogenesis were identified as DHs based on DNA markers (Doi *et al.* 2011). DH plants could be also obtained from haploids by chromosome doubling treatment using oryzalin. In *G. triflora*, Morgan *et al.* (2003) obtained a tetraploid plant from the diploid by treatment with oryzalin. Our results showed that oryzalin is also effective for production of DH and tetraploid plants with high frequency in varied gentian genotypes. Not only DH plants but also tetraploid plants are important for breeding new cultivars in gentian such as development of gigantic flowers and triploid hybrid cultivars.

The present study revealed that unfertilized ovule culture of gentians is a powerful tool for obtaining haploids and DHs because of its application to a wide range of genotypes and its reproducible and reliable nature. Not only breeding programs using regenerated plants obtained in this study, but also genetic and developmental studies of gynogenesis, are currently being carried out.

Acknowledgments

This work was supported in part by "Research and Development Projects for Application in Promoting New Policy of Agriculture, Forestry and Fisheries" from the Ministry of Agriculture, Forestry and Fisheries of Japan.

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